Applied Research Note: Pulsed-field gel electrophoresis and antimicrobial resistance profiles of *Campylobacter jejuni* isolated from Brazilian broiler farms

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Primary Audience: Poultry Veterinarians, Food Safety Specialists

SUMMARY

We studied the on-farm variety of thermotolerant *Campylobacter* strains isolated from field samples received over a 12-mo period from four broiler-producing companies in southern Brazil. In total, 515 of 807 (63.8%) of analyzed samples were found to be *Campylobacter*-positive. *Campylobacter jejuni* and *C. coli* were detected; the first one being more frequently isolated (98.2%). The highest antimicrobial resistance levels in a subset of 80 *C. jejuni* strains were found against enrofloxacin (89.5%) and ciprofloxacin (83.3%). Most of the 28 DNA macrorestriction profiles identified in *C. jejuni* were unique to each sampled broiler company. Genetically related *C. jejuni* populations were detected on different broiler farms integrated to the same company on different sampling occasions. Undistinguished strains were found in the indoor environment and in broilers on the same farm. The finding of recurrent strains on integrated farms suggests that particular factors or management practices may play a role in the dispersion or carry-over of *C. jejuni* in the studied broiler companies.

Key words: *Campylobacter*, poultry, antimicrobial resistance

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DESCRIPTION OF PROBLEM

Thermotolerant *Campylobacter* species are known to cause human foodborne enteritis, with fresh chicken being the most commonly implicated food type (Hansson et al., 2018). High loads of thermotolerant *Campylobacter* often colonize the intestinal tract of broilers, which may lead to carcass contamination at slaughterhouses (Prachantasena et al., 2016). Therefore, prevention of transmission to humans from chicken meat focuses on control strategies from farm to fork.

Efforts have been made to reduce *Campylobacter* in broiler production; however, complete eradication is currently unrealistic (Hansson et al., 2018). Hence, there is a need to better understand local *Campylobacter* epidemiology on broiler farms to establish proper preventive measures and keep the within-farm prevalence at low levels. Subtyping helps to identify the relationships between isolates within a flock as well as being useful to monitor geographic or temporal characteristics of strains (Prachantasena et al., 2016). In addition, antimicrobial susceptibility monitoring offers an overview on the strains resistance patterns with potential to spread from the food chain to

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humans (Abraham et al., 2020). Highly diverse *Campylobacter* strains have been detected in Brazil (Melo et al., 2019); nevertheless, the within-farm strain varieties remain unclear in broiler production. Here, we report the *Campylobacter* populations detected in samples received over a 12-mo period from Brazilian broiler farms integrated to different broiler-producing companies. This baseline study provides data to compare temporal changes on *C. jejuni* strains subtypes at farm level in southern Brazil that would be useful to identify farm-specific management practices to be validate for *Campylobacter* reduction in broiler chickens.

MATERIALS AND METHODS

Samples

Between 2010 and 2011, 807 field samples were received at Embrapa Swine and Poultry (Concórdia, SC, Brazil), a research unit of the Brazilian Agricultural Research Corporation (Embrapa), for thermotolerant *Campylobacter* diagnosis. Analyses were carried out using routine laboratory technique and with funding provided by the National Council for Scientific and Technological Development and the Brazilian Ministry of Agriculture, Livestock, and Food Supply to improve framework and resources of veterinary services (call no. 64/2008). Samples were sourced from four broiler companies in southern Brazil: A (1 farm, 32 samples); B (12 farms, 174 samples); C (1 farm, 521 samples); and D (7 farms, 80 samples). Individual farms were selected by the poultry veterinarians at each given broiler company. Drag swabs from broiler houses and swabs from broiler chicks transport crates were received in 150 mL of 1% buffered peptone water (**BPW**). Litter (∼ 500 g), feed (∼ 500 g) from feed hoppers, drinking water (∼ 500 mL) from drinking lines, darkling beetles collected from broiler houses, and paper lines from broiler chicks transport crates were individually provided in plastic bags. Broiler feces were placed in plastic bags with ∼ 3 times the volume of 1% BPW. Cloacal swabs were received in screw-cap tubes with Cary-Blair transport medium (Oxoid, Basingstoke, Hampshire, England). All samples were transported to the laboratory in insulated boxes with ice packs and processed within 24 h.

Campylobacter Detection

After mixing the content by hand, drinking water (10 mL), feed (10 g) and litter (10 g) samples were suspended in 90 mL of Bolton broth (Oxoid). Ten mL of BPW from drag swabs and swabs from transport crates were individually inoculated in 90 mL of Bolton broth. Macerated darkling beetles and paper lining were suspended in 1% BPW at a ratio of 1:4 and inoculated into nine times its volume of Bolton broth. Suspension of feces was mixed thoroughly, and 1 mL was inoculated in 9 mL of Bolton broth. Cloacal swabs were inoculated in 15 mL of Bolton broth. After incubation for 24 h at 41.5˚C in a microaerobic atmosphere (5% O_2, 10% CO_2, with balanced N_2; White Martins, Rio de Janeiro, RJ, Brazil), samples were concurrently streaked onto modified Charcoal Cefoperazone Deoxycholate agar (Oxoid), Preston agar (Oxoid) and Campy-Line agar (Oxoid) and incubated at 41.5˚C in the microaerobic atmosphere for 24 to 48 h. After microscopy, at least one presumptive colony was subcultured onto blood agar no. 2 (Oxoid) and Columbia agar (Merck, Darmstadt, Hesse, Germany) for 24 h at 41.5˚C and tested for oxidase (Newprov, Pinhais, Paraná, Brazil), catalase (Merck), hydrolysis of sodium hippurate (Inlab, São Paulo, São Paulo, Brazil) and indoxyl acetate (Sigma-Aldrich, St. Louis, MO). Briefly, a loopful of colonies was suspended in 0.4 mL of sodium hippurate solution and incubated for 2 h at 37˚C. Then, 0.2 mL of ninhydrin solution (Vetec, Duque de Caxias, Rio de Janeiro, Brazil) was added on the top of the sodium hippurate solution and incubated at 37˚C. A loopful of colony material was also placed on an indoxyl acetate disc and covered by a drop of sterile distilled water at room temperature. Sodium hippurate and indoxil acetate hydrolysis were checked after 10 min. According to the number of sampled farms at each broiler company, a subset of the isolated strains was selected to represent ∼15% of the detected *C. jejuni* strains, derived from the field materials that frequently tested positive for

Campylobacter and limited to a maximum of 10 strains from each integrated farm.

DNA was extracted from isolated colonies suspended in 0.5 mL of phosphate-buffered saline. A 0.1 mL aliquot was added to 40 μL of a suspension of diatomaceous silica (Sigma) and 0.9 mL of lysis buffer (5 M guanidinium thiocyanate [Sigma], 0.1 M Tris [Merck]-HCl pH 6.4, 0.2 M EDTA [Sigma] pH 8.0, 2.6% [v/v] Triton X-100 [Sigma]), mixed thoroughly and incubated at room temperature for 10 min. Suspension was centrifuged at 12,000 X g for 15 s and the pellet was washed twice in the washing buffer (5 M guanidinium thiocyanate, 0.1 M Tris-HCl pH 6.4), following by washes in 70% ethanol (Vetec) and acetone (Dinâmica, Indaiatuba, São Paulo, Brazil). DNA was resuspended in Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and quantified using the BioDrop (Biochrom, Waterbeach, Cambridge, England). Next, bacterial DNA was tested by polymerase chain reaction (**PCR**) based on a 287 bp sequence of the 16S rRNA gene from *C. jejuni, C. coli*, and *C. lari*. The PCR mixture contained the 1X GoTaq Flexi buffer (Promega, Madison, WI), 2.25 mM $MgCl_2$, 200 mM of each dNTP (Promega), 12 pMol of forward (5'-CTGCTTAACACAAGTTGAGTAGG-3') and reverse (5'-TTCCTTAGGTACCGTCAGAA-3') primers (IDT, Coralville, IA), 1 U of GoTaq DNA polymerase (Promega), and 15 ng of genomic DNA. Amplification was performed at 94˚C for 2 min followed by 35 cycles of 94˚C for 30 s, 60˚C for 15 s, and 72˚C for 30 s (Würfel et al., 2019). *Campylobacter jejuni* ssp. *jejuni* ATCC 33560 and *Campylobacter fetus* ssp. *venerealis* ATCC 19438 were used as positive and negative controls, respectively.

Genotyping and Antimicrobial Susceptibility

Genotyping was achieved using the well-standardized pulsed-field gel electrophoresis (**PFGE**) protocol validated by PulseNet for foodborne disease surveillance (Chinen et al., 2019). DNA macrorestriction patterns obtained after *Sma*I (New England Biolabs, Hitchin, Hertfordshire, England) digestion were analyzed using the BioNumerics software package, version 6.1 (Applied Maths, Sint-Martens-Latem, East Flanders, Belgium). Similarity was calculated by Dice percentage similarity coefficient with a position tolerance of 1.5%. A dendrogram was generated by cluster analysis using the unweighted pair group method with arithmetic averages (**UPGMA**). Antimicrobial susceptibility testing was performed by a microdilution procedure using cation-adjusted Mueller-Hinton broth (BD Difco, Detroit, MI) with 5% lysed horse blood as described (CLSI, 2010) to determine the minimum inhibitory concentrations (**MIC**) for ciprofloxacin (**CIP**), enrofloxacin (**ENO**), erythromycin (**ERI**), spectinomycin (**SPE**), and tetracycline (**TET**) (Sigma-Aldrich). *Campylobacter jejuni* ssp. *jejuni* ATCC 33560 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains. Interpretation used the criteria defined in the standards M45-A2 (CLSI, 2010) and M31-A3 (CLSI, 2008), where appropriate.

Statistical Analyses

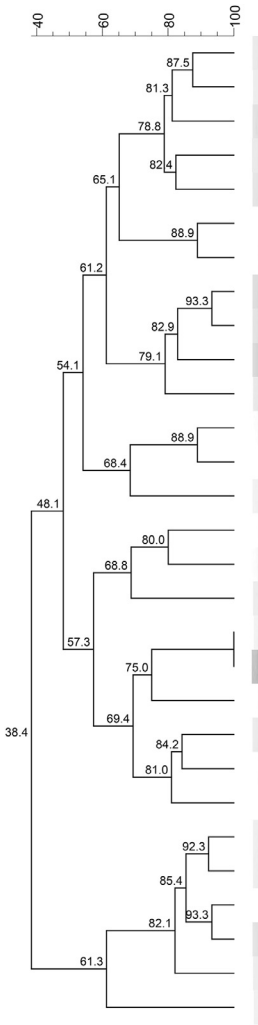
Statistical analyses were accomplished using SAS, version 9.4 (SAS Institute Inc., Cary, NC). Antimicrobial resistance (**AMR**) levels were individually compared using the chi-square test. Differences were evaluated using the variance analysis, which were compared by the Tukey test (5% significance level). Multiple correspondence analysis (**MCA**) was carried out using RStudio, version 1.2.5042 (RStudio Team, Boston, MA) to investigate associations among the most frequent PFGE and AMR patterns found in a minimum of five *C. jejuni* strains isolated from each broiler company.

RESULTS AND DISCUSSION

In total, 515 of 807 (63.8%) samples tested *Campylobacter*-positive, with 506 (98.2%) of the isolates identified as *C. jejuni* and 37 (7.2%) as *C. coli*. Twenty-eight samples tested simultaneously positive to both *Campylobacter* species. Positive samples were derived from company A (3/32), B (141/174), C (306/521) and D (65/80). Overall, 344 of 518 (66.4%) cloacal swabs; 65 of 83 (78.3%) drag swabs; 47 of 79 (59.5%) litter; 51 of 56 (91.1%) feces; 7 of 23 (30.4%) pooled darkling beetles; and 1 of 21 (4.8%) drinking water samples tested positive.

According to the criteria used to select the isolated strains, a subset of 80 *C. jejuni* strains comprising 3, 45, 10, and 22 strains from companies A, B, C, and D, respectively, derived from 20 farms, was analyzed for antimicrobial susceptibility and PFGE.

A total of 8 of 80 (10.0%) of analyzed *C. jejuni* strains were sensitive to all tested antimicrobials. Intermediate resistance was identified against enrofloxacin (5.0%) and ciprofloxacin (2.5%). Resistant strains were distributed among the following patterns: CIP-ENO-SPE-TET (38.7%), CIP-ENO (23.7%), CIP-ENO-ERI-SPE-TET (10.0%), CIP-ENO-SPE (3.7%), CIP-ENO-ERI (2.5%), ENO-SPE-TET (2.5%), CIP-ENO-ERI-SPE (2.5%), ENO (1.2%), and TET (1.2%). Resistant and sensitive strains were individually compared, revealing the highest resistance rates against enrofloxacin (89.5%) and ciprofloxacin (83.3%) ($P < 0.0001$), whereas the lowest resistance was found to erythromycin (15.0%, $P < 0.0001$). Strains showed moderate resistance to spectinomycin (57.5%, $P = 0.1797$) and tetracycline (52.5%, $P = 0.6547$), however, with no significant difference between each other. Respectively, macrolides and quinolones are among the critically important antimicrobials for



PFGE pattern	Company	Farm	Strain (no.)	Source
5	A	1	2	CS
4	B	2; 9	2	DS
8	D	6	5	CS; DS; L
6	A	1	1	CS
7	B	1	3	CS; DS
9	B	3	2	F; L
10	B	6	4	CS; L
25	B	2	1	DS
24	B	1; 2	8	CS; DS; F; L
27	B	2	1	F
26	B	2	2	CS; F
1	B	9; 10	4	CS; DS; L
2	B	10	1	CS
3	D	6	1	F
17	B	8	2	CS; DS
16	B	7	2	CS; DS
18	B	2	3	CS; L
20 *	B	3; 4	4	CS; F; L
20 *	C	1	10	CS; DS; L
19	B	5	2	CS; L
21	B	12	1	B
22	B	11	1	CS
23	B	11	1	DS
14	D	7	3	CS; F
15	D	7	1	L
11	D	2; 3; 4; 5	10	CS; DS; F; L
12	D	4	1	CS
13	D	7	1	F
28	B	12	1	B

Figure 1. Cluster analysis of PFGE patterns in a subset of the *Campylobacter jejuni* strains isolated from broiler companies (A-D). Asterisks indicate the common subtype found at different companies. Abbreviations: B, beetles; CS, cloacal swab; DS, drag swab; F, feces; L, litter.

human medicine, whose resistance in poultry-derived *Campylobacter* strains might limit the human campylobacteriosis therapy (Abraham et al., 2020). *Campylobacter* strains isolated from samples from broiler slaughterhouses have shown high levels of ciprofloxacin resistance in Brazil (Sierra-Arguello et al., 2018). On the other hand, resistance to macrolides is uncommon in *C. jejuni*; however, higher resistance rates to erythromycin have been reported in strains isolated from broiler carcasses (Melo et al., 2019).

Twenty-eight PFGE patterns were distinguished within analyzed *C. jejuni* strains (Figure 1). All strains from companies A and C were isolated from samples received from single broiler farms, reflecting epidemiologically linked sources. Notably, the PFGE-20 pattern identified in company C was also found in four nonrelated strains isolated from two farms in company B (Figure 1), which were detected on different sampling occasions, processed at the laboratory on different days, and mostly showed different AMR patterns (data not shown). The remaining genotypes identified comprised *C. jejuni* strains unique to each given sampled broiler-producing company.

Recurrent *C. jejuni* strains were detected in samples from different farms integrated to a given broiler company (B and D, Figure 1). Such an association was also shown by MCA, which analyzed the four most common PFGE and AMR patterns found in strains from companies B, C, and D (Figure 2). On the right of the resulting map, three farms integrated to company D were associated with both the PFGE-11 pattern and susceptibility to all tested antimicrobials. On the top left, two farms integrated to company B were associated to each other, according to PFGE-24 and CIP-ENO-SPE-TET patterns. Taken together, the results suggest the carry-over of strains from one farm to another or a common and persistent source of contamination at sampled farms. On the other hand, the CIP-ENO profile was closely related to farms integrated to companies C and D, showing association to two PFGE patterns, as revealed on the bottom left of the map.

Although data on antimicrobial use, either for prophylactic or therapeutic purposes, were unknown in the flocks from sampled farms, sensitive or resistant profiles would be most likely related to antimicrobial usage in flocks of studied companies. However, routes of resistance acquisition should be investigated. A recent

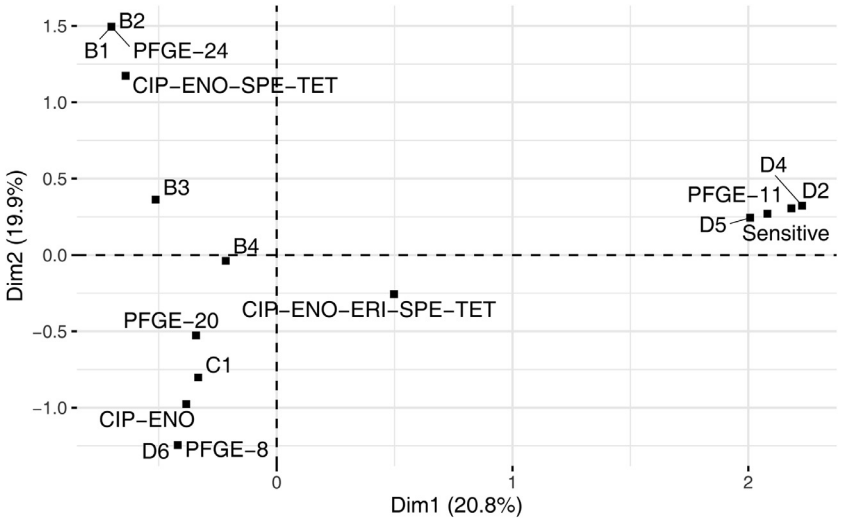


Figure 2. Multiple correspondence analysis describing the association among the most frequent PFGE and antimicrobial resistance patterns found in *Campylobacter jejuni* strains isolated from different farms integrated to broiler-producing companies (B, C, and D).

Australian study detected fluoroquinolone-resistant *Campylobacter* strains in commercial broiler flocks without evidence of direct selection pressure from the use of fluoroquinolones, most probably introduced after biosecurity failures at farms (Abraham et al., 2020).

Horizontal transmission from the environment still plays a major role in *Campylobacter* epidemiology in broiler farms (Hansson et al., 2018). Here, most of the *C. jejuni* strains isolated from environmental samples showed 100% similarity to the strains isolated from cloacal swabs on the same farm (Figure 1). It was not possible to determine the likelihood of either a contamination route from the indoor environment to the broilers or the inverse. Nevertheless, indoor environmental samples usually become contaminated after *Campylobacter* colonize broilers (Ellis-Iversen et al., 2012; Prachantasena et al., 2016). The alternative subtypes identified in strains isolated from the indoor environmental reveal minor *C. jejuni* populations without, however, having spread to the broilers. This finding indicates the ability of *C. jejuni* to survive within the broiler house and highlights the continuous contamination pressure.

Although routine cleansing and disinfection procedures at the downtime are generally efficient to decontaminate broiler houses where *Campylobacter*-positive flocks have been reared (Hansson et al., 2018), the predominance of distinct subtypes at each given broiler-producing company found in this study indicate that particular factors or management practices are involved in the persistence or carry-over of such strains on sampled farms. The results showed a high frequency of *C. jejuni* in samples analyzed, whose AMR levels were higher to enrofloxacin and ciprofloxacin. Such a finding highlights the need for further studies to investigate selective pressure for the development of fluoroquinolone resistance in *C. jejuni* strains on farms.

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DISCLOSURES

The authors declare that no competing financial interests exist.

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